Review: complement receptor 1 therapeutics for prevention of immune hemolysis

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The complement system plays a crucial role in fighting infections and is an important link between the innate and adaptive immune responses. However, inappropriate complement activation can cause tissue damage, and it underlies the pathology of many diseases. In the transfusion medicine setting, complement sensitization of RBCs can lead to both intravascular and extravascular destruction. Moreover, complement deficiencies are associated with autoimmune disorders, including autoimmune hemolytic anemia (AIHA). Complement receptor 1 (CR1) is a large single-pass glycoprotein that is expressed on a variety of cell types in blood, including RBCs and immune cells. Among its multiple functions is its ability to inhibit complement activation. Furthermore, gene knockout studies in mice implicate a role for CR1 (along with the alternatively spliced gene product CR2) in prevention of autoimmunity. This review discusses the possibility that the CR1 protein may be manipulated to prevent and treat AIHA. In addition, it will be shown in an in vivo mouse model of transfusion reaction that recombinant soluble forms of CR1 can reduce complement-mediated RBC destruction, thereby prolonging survival of transfused RBCs. It is proposed that CR1-based therapeutics have potential for effective and safe prophylactic short-term use and for treatment of hemolytic transfusion reactions.
***Immunohematology* 2005;21:109–118.**

Key Words: complement, complement receptor 1, sCR1, inhibitors, LHR, immune hemolysis, autoimmune hemolytic anemia, transfusion medicine, regulatory T cells, $CD4^+CD25^+$

The Complement System and Red Cell Destruction

The complement system is an important part of the innate immune system for fighting infections and foreign molecules before an adaptive response has developed.[1] It is also an important regulator of B-cell, and possibly T-cell, immunity.[2,3] However, it can also cause cellular and tissue damage when activated inappropriately, contributing to many clinical conditions, including reperfusion injury (following surgery, ischemic disease, and organ transplantation), organ rejection, acute inflammatory injury to the lungs, and autoimmune diseases.[1] For the system to exert its biological activities, it has to be activated. Activation occurs in a sequence that involves proteolytic cleavage of the complement components, resulting in the release of active biological mediators and the assembly of active enzyme molecules that result in cleavage of the next downstream complement component.[1] Depending on the nature of the activators, three complement activation pathways have been described: the antibody-dependent classical pathway and the antibody-independent alternative and lectin pathways (Fig. 1).[1] Common to all three pathways are two critical steps: the assembly of the C3 convertase enzymes and the activation of C5 convertases. Specifically, the C3 convertases cleave C3 into C3a, a potent anaphylatoxin, which acts as a neutrophil chemotaxin and activator,[4] and C3b, which covalently attaches to nearby targets, where it directs immune clearance and antigen selection.[5] In addition, the C5 convertases cause the release of another potent anaphylatoxin, C5a,[4] and lead to the formation of the pore-like membrane attack complex (C5b-9), which inserts into cell membranes, causing lysis or sublytic damage to the target cell (Fig. 1).[5]

In the transfusion medicine setting, complement-mediated RBC destruction plays a critical role, being involved in both intravascular and extravascular hemolysis.[6] Generally, in the presence of a potent, complement-binding antibody and large numbers of closely situated RBC antigens, complement activation can proceed to completion, resulting in intravascular hemolysis, which can be fatal.[7,8] However, the majority of blood group antibodies (including both alloantibodies and autoantibodies) that can fix complement activate complement up to the C3 stage but do not go on to act as hemolysins.[9] Although antibody-coated RBCs can also be destroyed extravascularly without complement activation, RBC removal by tissue

macrophages in the spleen and liver is enhanced considerably when C3 is present on RBCs in addition to IgG.[9,10] Indeed, as many as 50 percent of patients with autoimmune hemolytic anemia (AIHA) have both IgG and complement on their RBCs.[11]

Complement Regulatory Proteins and CR1

The complement system can be divided into three separate pathways (classical, alternative, and lectin), depending on the type of activator. The system consists of nearly 30 different serum and membrane proteins which, after activation, interact in a highly regulated enzymatic cascade to generate reaction products that mediate inflammation and host protection.[1] Because of the direct and indirect powerful cytolytic activity of complement, there exists a family of structurally and functionally related proteins, known as regulators of complement activation (RCA), that prevent potential host cell damage from complement activation (Fig. 1).[5] CR1, also known as CD35, is the most versatile of the RCA family because it exhibits decay-accelerating and cofactor properties that can inactivate the two critical enzymes of the complement activation pathways (Fig. 1 and Fig. 2).[12–15] Specifically, by binding to C4b or C3b, CR1 can displace the catalytic subunits (decay-accelerating activity [Fig. 2]) of the convertases. In addition, by acting as a cofactor for plasma protease factor I, CR1 is responsible for the degradation of C4b and C3b (Fig. 2), and thus complete inactivation of the convertase. CR1 has also been shown to function as a receptor for C1q,[16] the first component of the classical pathway as well as the mannan-binding lectin (MBL) of the lectin pathway.[17]

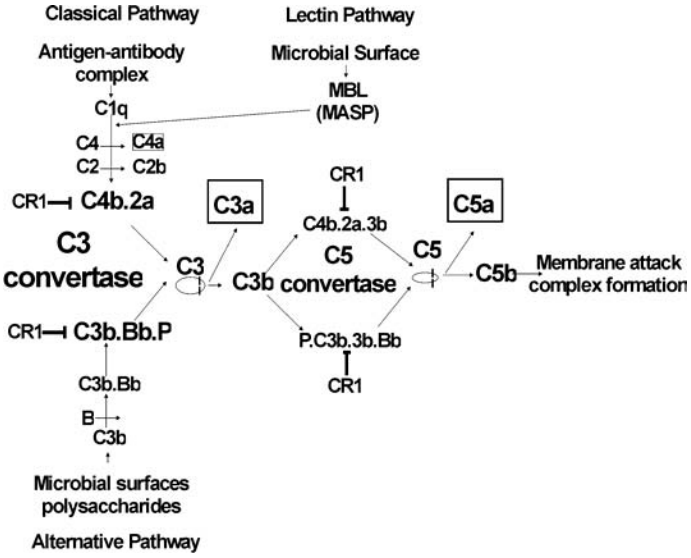

Fig. 1. The complement system. The classical pathway is activated by antigen-antibody complex and the alternative and lectin pathways by microbial surfaces. Activation of these pathways results in the generation of the key enzymes, C3 and C5 convertases, which in turn results in the release of C3a, C4a, and C5a anaphylatoxins (inflammatory response), C3b (opsonization of target cells), and the generation of the membrane attack complex in the target cell (lysis). The different steps of the cascade where CR1 inhibits complement activation are shown. Abbreviations: MBL, mannan-binding lectin; MASP, MBL-associated serine protease.

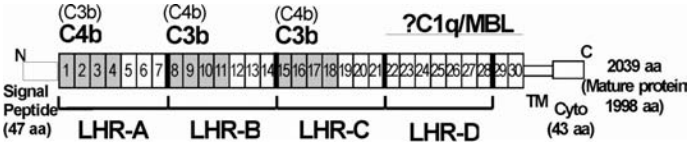

Fig. 2. Regulation of complement by decay-accelerating and cofactor activitiy of CR1. The classical complement inhibitory activity of CR1 is depicted. CR1 dissociates C3 convertase of the classical pathway through its decay-accelerating activity. In addition, through its cofactor activity, it degrades C4b into C4c and C4d. Central to these activities is its ability to bind C4b. (Adapted from Hourcade et al.[12])

CR1 Expression Pattern and Functions

CR1 is expressed on a number of cell types, mostly in the blood, and at low levels in soluble form in the plasma.[18] Of the C4 and C3 regulatory proteins, CR1 has the widest range of activities and functions, including immune complex clearance, regulation of complement activation, phagocytosis, and antibody response, as well as deletion of autoreactive B and maybe T cells. Through its ability to bind C3b and C4b, erythrocyte CR1 transports immune complexes to the spleen and liver for their removal[19–23]; CR1, expressed on macrophages and neutrophils, mediates adherence and phagocytosis[24,25]; on B cells, CR1 modulates the threshold for B-cell activation[26,27]; and on follicular dendritic cells, it is thought to improve immune response to antigen.[28–36] Through its ability to bind C3b and C4b, erythrocyte CR1 transports immune complexes to the spleen and liver for their removal,[19–23] CR1 expressed on macrophages and neutrophils mediates adherence and phagocytosis,[24,25] that expressed on B cells modulates the threshold for B-cell activation,[26,27] and that on follicular dendritic cells is thought to improve the immune response to antigen.[28–36] Moreover, CR1 is expressed on 10 to 15 percent of human T lymphocytes,[37–40] although its function on such cells is not clear. The expression pattern of murine CR1 is for the most part similar to that of human CR1 except that mouse RBCs do not express CR1.[41] To date, no individuals completely

lacking the CR1 protein have been identified. However, RBCs that express low copy numbers of CR1 and its associated Knops blood group antigens, known as the Helgeson phenotype, have been described and appear to be associated with protection against severe malaria.[42]

CR1 Structure

In humans, four allotypes of CR1, varying in size, are known.[43] The predicted amino acid sequence of the common allotype (CR1*1) is 2039 residues (Fig 3).[18,44,45] The extracellular 1930-residue–long domain of CR1, with 25 potential N-glycosylation sites, can be divided into 30 short consensus repeats (SCRs), each of 59 to 72 amino acids (aa) with sequence homology between SCRs ranging from 60 to 90 percent.[45] Homologous SCRs with four conserved cysteine residues are also found in other RCA members.[12] The first 28 SCRs of CR1 are further arranged into four longer regions of similarity, termed long homologous repeats (LHRs A through D), consisting of seven SCRs each[18,44] (Fig. 3). In mice, CR1 is expressed along with the alternatively spliced gene product CR2.[46–48] Murine CR1 consists of 21 SCRs, with 15 SCRs identical to murine CR2 and 6 unique SCRs at the amino terminus.[46–48]

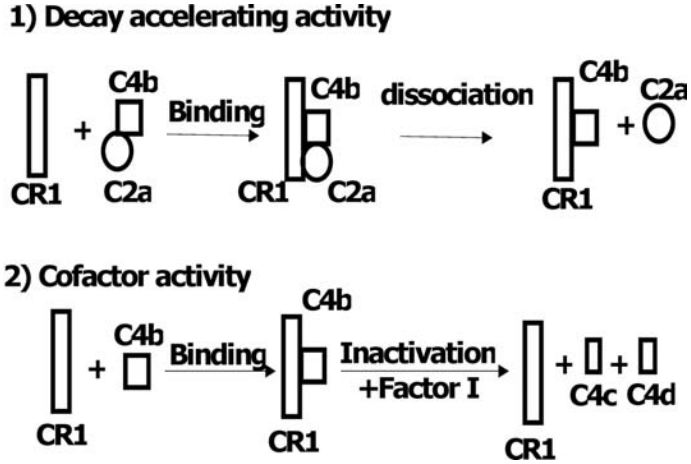

Fig. 3. Schematic representation of the common isotype of CR1. The 30 SCRs are represented by blocks. Groups of seven SCRs are further subdivided into four LHRs. The ligand binding sites for C3b and C4b are shaded. The preferred ligand is in bold; the alternative ligand is in parentheses. The positions of C1q/MBL binding sites have not yet been defined.

CR1-Based Therapeutics

The complement regulatory function of CR1 has been exploited for development of a potent anti-complement agent. Specifically, a recombinant soluble form of CR1 (sCR1), by binding C3b and C4b and inactivating the convertases, has successfully inhibited the complement activation cascade and prevented complement-mediated tissue injury in several animal models.[49,50] More importantly, sCR1 has been in human clinical trials for the treatment of acute respiratory distress syndrome and to reduce tissue damage in myocardial infarction and lung transplantation,[51–53] with possible favorable outcomes.[52] Despite the role of CR1 as a global inhibitor of complement activation, the antibacterial defenses of the patients undergoing treatment have not, thus far, been compromised, underscoring the usefulness of sCR1 as a therapeutic agent.[53] It is important to note that there are currently no anticomplement therapeutics in the clinic. Indeed, sCR1 represents the best in vivo characterized anticomplement agent to date.

Our studies are the first to demonstrate a potential for sCR1 for inhibiting complement-mediated RBC destruction following transfusion immunization events.[53,54] Furthermore, through structure-function analysis we have identified a 254-aa domain at the N-terminus of CR1, consisting of four SCRs and one-eighth of sCR1, that has antihemolytic activity in vivo. Previous in vitro studies by us and others demonstrated that the N-terminal domain of CR1 has barely any cofactor activity, but has decay-accelerating activity for the C3 convertases and inhibits the classical activation pathway.[54,56–61] Moreover, we and others have shown that the four N-terminal SCRs preferentially bind C4b.[45,56,59,62–64] Based on these observations, we believe that fine mapping of the C4b binding and the decay-accelerating activity (C4b.2a) for the classical activation pathway in the 254-aa domain will lead to future design of nonimmunogenic small molecule inhibitors to down-regulate complement-mediated RBC destruction. It is important to note that chronic treatment with complement inhibitors is likely to undermine the body's ability to fight infections[65] and may lead to development of autoimmune diseases (discussed in a later section). The application for these inhibitors is thus for short-term prophylactic use before transfusion of not fully matched blood in emergency situations and as a therapeutic option in select patients with complement-mediated immune hemolysis to ameliorate the life-threatening complications.

Complement and AIHA

AIHA is an autoimmune disease caused by autoantibodies against RBC self-antigens, causing

shortened RBC survival.[6] The underlying mechanism for breakdown of immunologic tolerance is not well established. Several lines of evidence support the role of complement in the maintenance of tolerance to self-antigens: (1) Hereditary deficiencies of complement proteins of the classical pathway have been associated with autoimmune diseases.[66] For example, 90 percent of C1q-deficient patients develop autoimmune disease,[67] and C1q knockout mice have a high incidence of developing autoimmune disease.[68] (2) Altered levels of expression of CR1 have been observed in patients with AIHA as well as other autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, Sjogren's syndrome, and some diabetic patients.[69-77] In a mouse model of severe lupus-like disease (MRL/lpr), lower levels of CR1/CR2 receptors have been found on B cells before the development of disease manifestations,[78] suggesting that altered complement receptor expression may contribute to initiation or progression of autoimmune disease. Gene knockout studies of mice lacking CR1 (along with the alternatively spliced gene product CR2) indicate that CR1 and CR2 control the activation thresholds of B cells to self-antigens,[79] although the exact molecular mechanism is not clearly understood. Because CR1/CR2 knockout mice lack both CR1 and CR2, the specific contribution of each receptor cannot be dissected. However, mice deficient in C4, but not C3, have a phenotype similar to CR1/CR2-deficient mice in studies demonstrating their role in immune tolerance, strongly suggesting that the primary effect in these mice is mediated by CR1.[79] (3) C3b was recently shown to induce the development of T-regulatory cells, known to be important for maintenance of peripheral tolerance (discussed in a later section). Specifically, it was shown that membrane cofactor protein (MCP, CD46), a natural complement regulatory protein whose ligands are C3b and C4b,[80] can act as a coreceptor for inducing the development of IL-10–secreting $CD4^+CD25^+$ regulatory T cells, which are responsible for active suppression of autoreactive T cells.[81]

T-Regulatory Cells

T-regulatory cells (Tregs) are a subset of T cells that function to control immune responses, including those directed against self-antigens. Different populations of Tregs have been described, including thymically derived naturally occurring cells and those that are induced in the periphery through exposure to antigen.[82,83] Naturally occurring Tregs constitute about 1 to 2 percent of peripheral blood mononuclear cells, or about 5 to 10 percent of the $CD4^+$ T cells, and are characterized by coexpression of CD25 (α subunit of IL-2 receptor) and the transcriptional repressor FoxP3 (forkhead box P3).[84] Their role in maintenance of self-tolerance and their ability to suppress a number of autoimmune diseases has attracted a great deal of attention and opened the possibility of developing novel immunotherapeutic strategies for suppression of autoimmunity.[84,85] Nevertheless, many questions remain to be answered about the characteristics and biology of Tregs. For example, it is not known whether the suppressive activity of the $CD4^+CD25^+$ Tregs can be subdivided to smaller subpopulations or whether smaller individual $CD4^+CD25^+$ Tregs have different degrees of suppressive activity.[86] Supporting this possibility, $CD103^+$ T cells within $CD4^+CD25^+$ Tregs were shown to have more suppressive activity than $CD103^-CD4^+CD25^+$ Tregs and $CD62L^{high}$ expressing $CD4^+CD25^+$ Tregs appear more potent in preventing certain autoimmune diseases in mice.[87,88] Preliminary studies in my laboratory indicate that CR1 is indeed expressed on a subpopulation of $CD4^+CD25^+$ Tregs. The molecular basis of the mechanism of Treg cell-mediated suppression is not fully known, although the consensus is that they can expand and augment their suppressive activity when stimulated.[89,90] In vitro naturally occurring $CD4^+CD25^+$ mediate suppression of cocultured CD25- T cells by cell-cell interactions, not by cytokines.[91-94] Activation has been shown to occur through TCR-specific signals.[90,95-97] Interestingly, non-TCR–specific stimuli such as bacterial products through Toll-like receptor 4 have also been shown to activate $CD4^+CD25^+$ Tregs.[98] Given that innate immune responses such as Toll-like receptors can stimulate Tregs, it is conceivable, although as yet untested, that complement activation products, through interactions with complement receptors such as CR1, may also be involved in augmenting or attenuating the activation state of Tregs, with consequences for their suppressive activity. In support of this possibility, C3b-CD46 interactions were recently shown to activate a subset of Tregs, namely the inducible Tregs.[81] Preliminary studies from my laboratory indicate that CR1 can also mediate the suppressive activity of $CD4^+CD25^+$ Tregs, although the exact mechanism of suppression is still under investigation.

Possible Role of Complement/CR1 in Treg Induction

We speculate that after certain infections, complement is activated and foreign antigens that are opsonized with complement components C3b and C4b, which are known to be the ligands for CR1 and CD46, may induce regulatory T cells. Thus, the initial complement activation will help clear the pathogen by direct lysis, inflammatory response, phagocytosis of complement-sensitized infectious agent, or a combination of these (Fig. 1). In our hypothetical model, complement activation through induction of Tregs will also result in suppression of certain self-reactive lymphocytes as well as effector T cells that would normally cause infection-induced immunopathology (Fig. 4). Given that complement is so tightly regulated,[1] it is conceivable that many factors, including the nature and strength of the activators (pathogens), will determine the balance among complement activation, Treg induction, and T-cell suppression. Our model obviously does not preclude the role of other mediators besides complement activation products for Treg activity, which may explain why certain infections persist or become chronic[99] and are correlated with lower incidence of autoimmunity.[100] Nevertheless, complement deficiencies, which we propose can cause decreased Treg activity, are associated with autoimmune disease.[67,101] AIHA appears to be a secondary complication in patients with a range of diseases, including those with viral or mycoplasmal infections,[102] and the presence of cross-reactive foreign antigens may be the underlying mechanism for breakdown of tolerance in these patients.[103] It is interesting to note that Rh antibodies do not fix complement on RBCs. In warm-type AIHA, many of the autoantibodies are directed against the Rh complex.[6] It may be that antigens such as Rh that are not initially opsonized with complement components may not be subject to immune tolerance. Thus, upon stimulation with cross-reactive foreign antigens, autoreactive T cells specific to Rh antigens are preferentially expanded, resulting in AIHA. Future studies are needed to further explore the potential contribution of CR1 and its ligands in the development of AIHA.

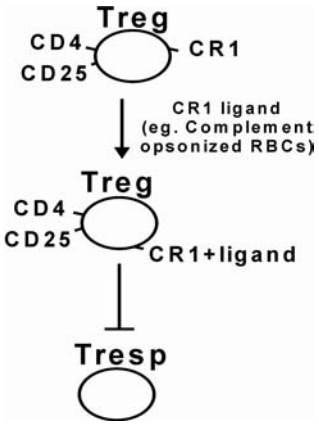

Fig. 4. Hypothetical role of CR1 in suppression of autoreactive T cells. Stimulation of Tregs with CR1 ligands such as complement opsonized RBCs results in suppression of Tresp (autoreactive or effector T cells). CR1-mediated activation signals described in different cell types may be involved in Treg activation.[39,40]

Acknowledgments

The author was supported in part by grants from the NIH R01 HL69102 and the American Heart Association Grant-in-Aid Heritage Affiliate.

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